Inhibition of Hexokinase and Protein Kinase Activities of Tumor Cells by a Chloromethyl Ketone Derivative of Lactic Acid†

John H. Johnson, Andrzej Zimniak,‡ and Efraim Racker*

ABSTRACT: A chloromethyl ketone derivative of lactic acid is a potent inhibitor of glycolysis of Ehrlich ascites tumor cells. It inhibited glycolysis of intact cells by about 50% at 200 μM (100 nmol/mg of protein) while cell-free extracts were inhibited 50% at 50 μM (50 nmol/mg of protein). N^{α}-(*p*-Tosyl)-L-lysine chloromethyl ketone and N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone inhibited only slightly or not at all at this concentration. The inhibition was localized at the hexokinase and phosphofructokinase steps since these two enzymes added to an inactivated extract restored the glycolytic activity, whereas none of the other glycolytic enzymes did. In fact, addition of pyruvate kinase or lactate dehydrogenase, which stimulated glycolysis, resulted in a more pronounced inhibition. Glycolysis and hexokinase activities in extracts of Rous sarcoma virus transformed cells were considerably more sensitive to the inhibitor than the activities from normal chick embryo fibroblasts. Hexokinase from mouse brain required 50 times higher concentrations for inhibition than the enzyme from mouse Ehrlich ascites tumor cells. Yeast hexokinase was unaffected at all concentrations tested. Since 5,5′-dithiobis(2-nitrobenzoate) protected against the inhibition, the chloromethyl ketone appeared to inhibit by interaction with an essential SH group. A pronounced inhibition of protein kinase activity of plasma membranes of Ehrlich ascites tumor cells was observed in the presence of the chloromethyl ketone. As in the case of glycolysis, the chloromethyl ketone of lactic acid was a more potent inhibitor of protein kinase activity than several other chloromethyl ketones that were tested.

A chloromethyl ketone derivative of lactic acid has been synthesized with the hope that it might be suitable as a suicide inhibitor by interfering with the excretion of lactic acid in actively glycolyzing cancer cells. Actually, a mixed anhydride of lactic acid formed during the synthesis was found to be an excellent inhibitor of lactic acid excretion (Johnson et al., 1980), but the chloromethyl ketone derivative was inactive. On the other hand, it was observed that the chloromethyl ketone was a potent inhibitor of glycolysis of Ehrlich ascites tumor cells and it impaired the growth of several cell lines.[1] In this paper we describe the effect of this and of three other chloromethyl ketone derivatives on glycolysis of cells and cell extracts, on a variety of hexokinase preparations, and on protein kinase activity of plasma membranes of Ehrlich ascites tumor cells.

Experimental Procedures

Materials. Isobutyl chloroformate, *N*-methylmorpholine, and tetrahydrofuran were obtained from Aldrich Chemical Co., Milwaukee, WI. Lactic acid, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone, and N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone were obtained from Sigma Chemical Co., St. Louis, MO. Glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-diphosphate were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Yeast hexokinase was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. 1-(*O*-Benzoyl)-3-chloro-2-propanone was the generous gift of Dr. John Kozarich, Yale University, and was subsequently synthesized as previously described (Hartman, 1970).

Synthesis of 2-Isobutyl 3-Oxo-4-chloro-2-butyl Carbonate. The synthesis of 2-isobutyl 3-oxo-4-chloro-2-butyl carbonate was performed essentially as described earlier for amino acid chloromethyl ketones (Powers, 1977) except that lactic acid (free acid) was desiccated over P_2O_5 for 48 h prior to use and tetrahydrofuran was redistilled over $LiAlH_4$.

Purification of iLac-CH_2Cl[2] was achieved by placing the oil on a 35 × 2 cm column packed with silica gel 60 and eluting with chloroform. Fractions containing iLac-CH_2Cl were found to be a light, colorless oil and were eluted closely behind the void volume. Purity was >95% as judged by proton magnetic resonance (Figure 1).

The purified material was applied to silica gel 60 thin-layer chromatography plates and developed in $CHCl_3$. A single spot (R_f 0.75) was detected by H_2SO_4 charring. A mass spectrum of the material revealed a parent peak at *m/e* 223 and a base peak at *m/e* 57 (Figure 2). The calculated molecular weight of iLac-CH_2Cl is 222.7.

Cell Cultures and Extract Preparation. Ehrlich ascites tumor cells were harvested 7–10 days after injection into male Swiss white mice. Following dilution in a buffer containing 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes), pH 7.4, 105 mM NaCl, 5 mM KCl, 4 mM NaP_i, and 1 mM $MgCl_2$, the cells were sedimented at 4 °C by centrifugation at 800*g* for 5 min, resuspended in the same buffer, incubated at 30 °C for 10 min, and then resedimented once at room temperature and once at 4 °C by centrifugation at 800*g* for 5 min.

Culturing of chick embryo fibroblasts and transformation of chick embryo fibroblasts with Rous sarcoma virus has been

† From the Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853. *Received November 9, 1981.* This investigation was supported by Grant BC-156 (E.R.) from the American Cancer Society and Grants CA-14454 (E.R.) and CA-08964 (E.R.) and Fellowship F32-CA06336 (J.H.J.) awarded by the National Cancer Institute, Department of Health and Human Services.

‡ Present address: Organic Chemistry and Technology, Warsaw Institute of Technology, Warsaw, Poland.

[1] J. H. Johnson, unpublished observations.

[2] Abbreviations: iLac-CH_2Cl, 2-isobutyl 3-oxo-4-chloro-2-butyl carbonate; BCP, 1-(*O*-benzoyl)-3-chloro-2-propanone; Tos-Lys-CH_2Cl, N^{α}-(*p*-tosyl)-L-lysine chloromethyl ketone; Tos-Phe-CH_2Cl, N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone; DTNB, 5,5′-dithiobis(2-nitrobenzoate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; ATP, adenosine 5′-triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; cAMP, adenosine 3′,5′-phosphate; EAT, Ehrlich ascites tumor cells.

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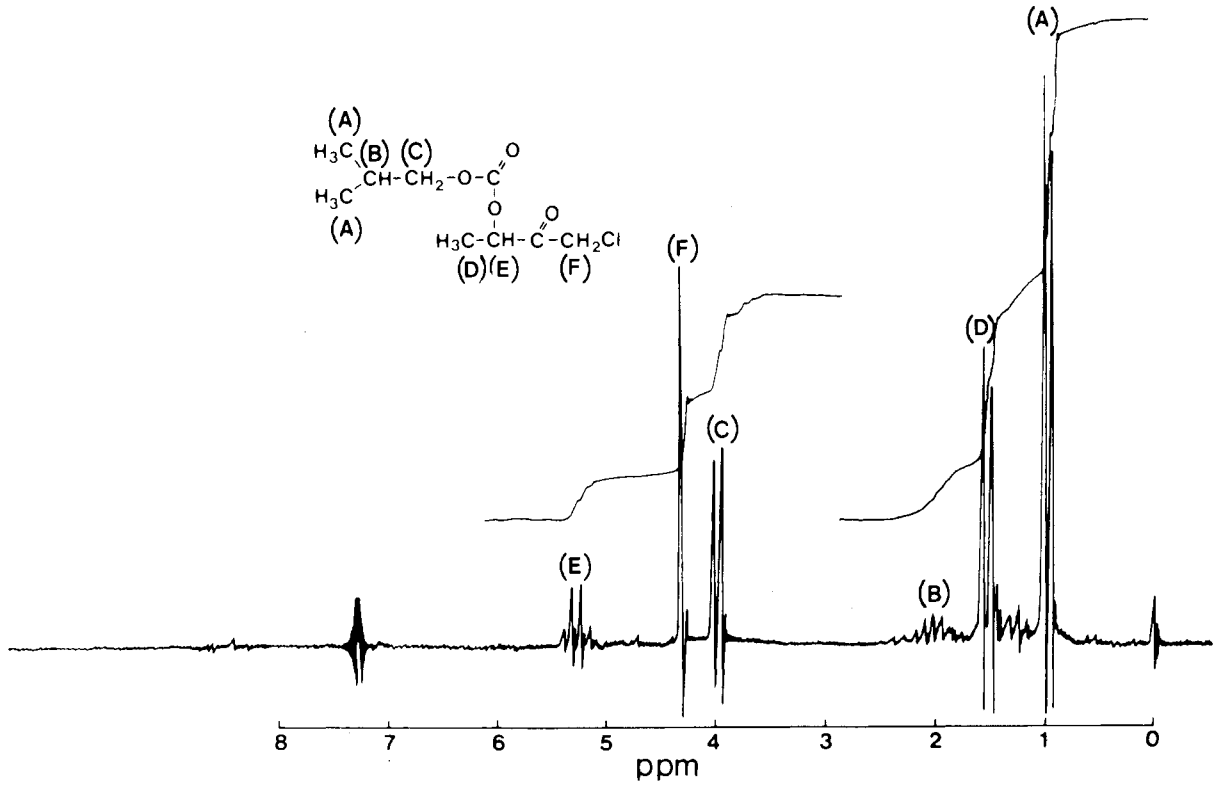

FIGURE 1: Proton magnetic resonance spectrum of 2-isobutyl 3-oxo-4-chloro-2-butyl carbonate. The spectrum was obtained in a $CDCl_3$ solvent with tetramethylsilane as an internal lock standard at 90 MHz.

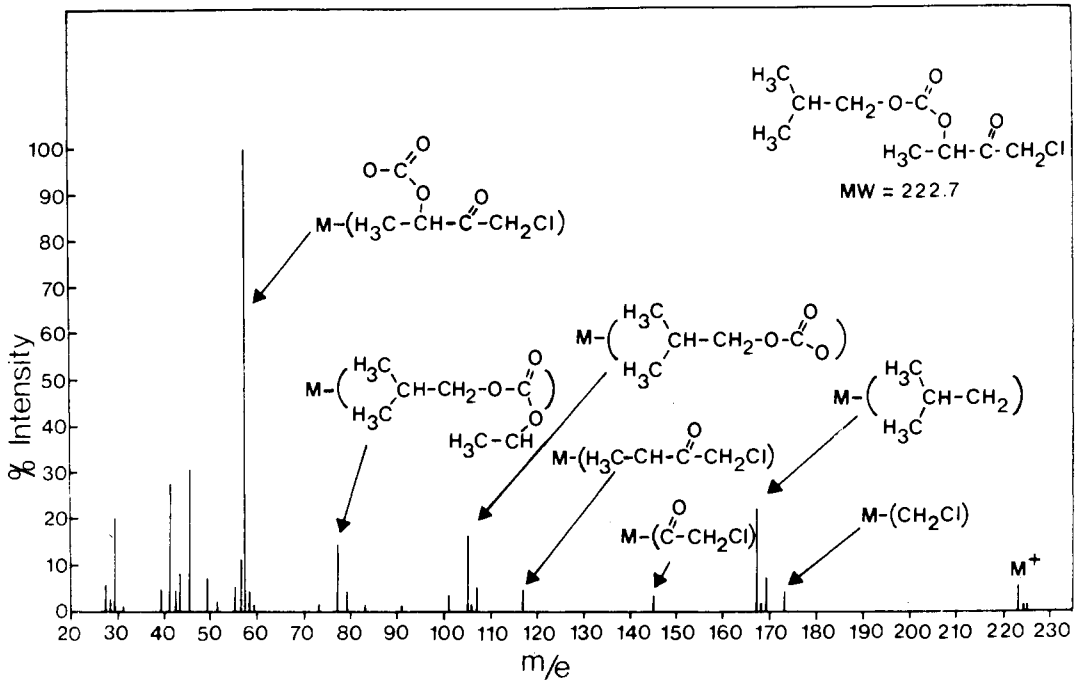

FIGURE 2: Electron impact mass spectrum of iLac-CH_2Cl. The spectrum was obtained on a MS902/C15-2 spectrometer coupled to a V-6 Datasystem 2040 analyzer.

described elsewhere (Fagan & Racker, 1978). Secondary cultures were harvested the third day following inoculation and washed as described above.

Extracts were prepared by resuspending the cells to approximately 0.5 g wet wt/mL of 2 mM EDTA, pH 7.0. Following a 5-min homogenization with acid-washed glass beads (Sigma, 75–150 μM), unbroken cells and nuclei were removed by centrifugation at 800g for 5 min at 0 °C. The supernatant was collected and centrifuged at 150000g for 15 min at 0 °C. The supernatant was again collected and stored at −70 °C until use.

Glycolysis Assays. Glycolysis assays were performed essentially according to the procedure of Wu & Racker (1959). Cells were resuspended in the Hepes, pH 7.4, buffer described above to a density of 2 mg of protein/mL. Chloromethyl ketones were added as ethanolic solutions at the concentrations indicated in the text (1% final ethanol concentration), and the suspensions were incubated for 15 min at 37 °C with shaking in a Dubnoff metabolic shaking incubator. Controls were assayed in the presence and absence of ethanol in each case, and no adverse effects of ethanol were observed. Glucose was then added to 10 mM final concentration. Following a 30-min

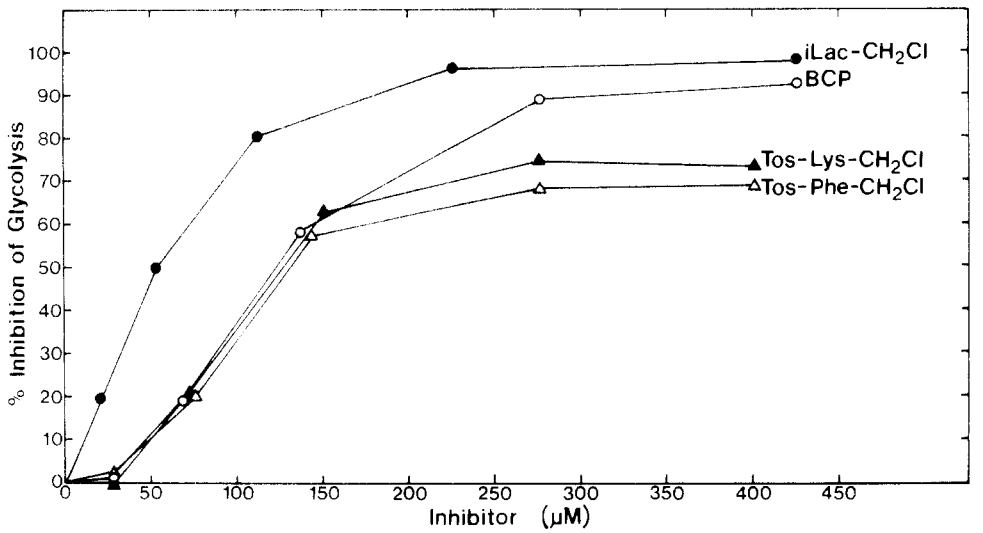

FIGURE 3: Inhibition of glycolysis in extracts from Ehrlich ascites tumor cells by iLac-CH_2Cl (●), BCP (○), Tos-Lys-CH_2Cl (▲), and Tos-Phe-CH_2Cl (△). Extract preparation, treatment with chloromethyl ketones, and glycolysis measurements are described under Experimental Procedures. The rate of glycolysis in the control extract was 0.46 μmol of lactate (30 min)$^{-1}$ (mg of protein)$^{-1}$.

incubation with shaking, 2 N perchloric acid (400 μL/mL of suspension) was added, and the reaction chambers were placed on ice. Lactic acid was determined according to the procedure of Hohorst (1963).

Glycolysis in extracts was measured by using essentially the same procedure except that 1 mg of extract protein/mL of Hepes buffer, supplemented with 20 mM NAD and 5 mM ATP, was used and the incubation was at 30 °C without shaking.

Hexokinase Assays. Measurements of hexokinase activity were made by coupling the formation of glucose 6-phosphate to glucose-6-phosphate dehydrogenase and monitoring the reduction of NADP at 340 nm according to the procedures of Slein (1963). Mouse brain hexokinase was prepared according to the procedure of Crane & Sols (1955). Hexokinase from Ehrlich ascites tumor cells, cultured chick embryo fibroblasts, and Rous sarcoma virus chick embryo fibroblasts was assayed in the cell-free extract. Membrane-bound hexokinase from these sources was obtained by centrifuging the membrane fraction obtained in the extraction procedure at 800*g* for 15 min to remove nuclei. Solubilization of membrane-bound hexokinase from brain and Ehrlich ascites tumor cells was accomplished by incubating the fractions in 2 mM EDTA, pH 7.0, containing 10 mM ATP at 0 °C for 20 min (Wilson, 1968). Membrane material was then removed by centrifugation at 150000*g* for 15 min at 0 °C.

Inhibitors were added at the indicated concentrations and incubated with the enzyme for 15 min at 0–5 °C. A 5-min temperature equilibration period was routinely allowed, and assays were performed at room temperature.

Assays of Membrane-Bound Protein Kinase Activity. Plasma membranes from Ehrlich ascites tumor cells were prepared by using a modification (Hinnen et al., 1979) of the method of Brunette & Till (1971). Membranes (100 μg of protein) were incubated in 100 μL of buffer containing 20 mM potassium phosphate, pH 7.0, 5 mM $MgCl_2$ and 1 mM [γ-^{32}P]ATP (100 cpm/pmol). The reaction was terminated by applying 50 μL of the reaction mixture to a Whatman 3MM filter paper and placing the filter papers in a solution of ice-cold 10% trichloroacetic acid. This was followed by four washes in 10% trichloroacetic acid, one wash in 95% ethanol, and one wash in ether according to the procedure of Vardanis (1977). The dried filter papers were then placed in scintillation vials containing 12 mL of Liquiscint, and the radioactivity was measured with a liquid scintillation spectrophotometer.

Table I: Inhibition of Glycolysis in Ehrlich Ascites Tumor Cells and a Cell-Free Extract from Ehrlich Ascites Tumor Cells by 2-Isobutyl 3-Oxo-4-chloro-2-butyl Carbonate[a]

preparation	[iLac-CH_2Cl]	μmol of lactate (30 min)$^{-1}$ (mg of protein)$^{-1}$	% inhibition
whole cells	none	0.470	
	200 μM	0.250	47
cell-free extract	none	0.481	
	100 μM	0.110	77

[a] Treatment of cells, preparation of cell-free extracts, and assays of glycolysis are described under Experimental Procedures. The concentration of iLac-CH_2Cl was 100 nmol/mg of protein in the experiment with cells as well as with extract.

Other Methods. Protein was determined according to Lowry et al. (1951) after solubilization in 1% deoxycholate. [γ-^{32}P]ATP was prepared as described elsewhere (Avron, 1961).

Results

As shown in Table I, the chloromethyl ketone of lactate (iLac-CH_2Cl) is a potent inhibitor of glycolysis of Ehrlich ascites tumor cells. With intact cells about 50% inhibition was observed at 200 μM or 100 nmol/mg of protein. Cell-free extracts were 75–100% inhibited at this concentration. Since no "normal" control cells for Ehrlich ascites tumor cells are available, we compared the effect of iLac-CH_2Cl on chick embryo fibroblasts and cells transformed by Rous sarcoma virus. As shown in Table II, intact chick cells were slightly affected by low concentrations of iLac-CH_2Cl, whereas the transformed cells showed a sensitivity similar to that of EAT cells. Interestingly, a similar though less pronounced difference in sensitivity was observed between the cell-free extracts.

As shown in Figure 3, a comparison of four different chloromethyl ketone derivatives showed significant differences in their effects on glycolysis in the tumor cell extracts. A 50% inhibition was observed at about 50 μM iLac-CH_2Cl, whereas about 3 times higher concentrations were required for the same inhibition in the presence of Tos-Phe-CH_2Cl or Tos-Lys-CH_2Cl. BCP was observed to inhibit 50% at 150 μM.

So that the site of inhibition of glycolysis in the cell-free extract could be located, purified glycolytic enzymes were

Table II: Inhibition of Glycolysis in Whole Cells and Extracts of Chick Embryo Fibroblasts and Rous Sarcoma Virus Chick Embryo Fibroblasts by 2-Isobutyl 3-Oxo-4-chloro-2-butyl Carbonate[a]

preparation	[iLac-CH_2Cl]	μmol of lactate (30 min)$^{-1}$ (mg of protein)$^{-1}$	% inhibition
fibroblasts	none	0.162	
	200 μM	0.153	6
transformed fibroblasts	none	0.366	
	200 μM	0.155	58
fibroblast extract	none	0.171	
	100 μM	0.125	27
transformed fibroblast extract	none	0.422	
	100 μM	0.134	68

[a] Growth conditions, treatment of cells, preparation of extracts, and assays of glycolysis are described under Experimental Procedures. The concentration of iLac-CH_2Cl was in all cases 100 nmol/mg of protein.

Table III: Effects of Adding Various Enzymes of Glycolysis to Extracts of Ehrlich Ascites Tumor Cells Pretreated with 2-Isobutyl 3-Oxo-4-chloro-2-butyl Carbonate[a]

addition	[iLac-CH_2Cl]	μmol of lactate (30 min)$^{-1}$ (mg of protein)$^{-1}$	% inhibition
none	none	0.430	
none	50 μM	0.185	57
hexokinase	none	0.810	
	50 μM	0.593	27
phosphofructokinase	none	0.882	
	50 μM	0.655	36
aldolase	none	0.795	
	50 μM	0.330	58
triosephosphate isomerase	none	0.496	
	50 μM	0.230	54
glyceraldehyde-3-phosphate dehydrogenase	none	0.482	
	50 μM	0.255	47
phosphoglycerate kinase	none	0.585	
	50 μM	0.264	55
phosphoglycerate mutase	none	0.460	
	50 μM	0.215	53
enolase	none	0.431	
	50 μM	0.250	42
pyruvate kinase	none	0.769	
	50 μM	0.122	85
lactic dehydrogenase	none	0.819	
	50 μM	0.247	70
hexokinase and phosphofructokinase	none	1.223	
	50 μM	1.059	13

[a] The extract was incubated with iLac-CH_2Cl at 30 °C for 15 min. Then 0.5 unit of each individual enzyme listed was added immediately prior to the addition of glucose. The iLac-CH_2Cl concentration was 50 nmol/mg of protein of extract.

added individually and in combination to the glycolyzing extract. As can be seen from Table III, the addition of hexokinase and phosphofructokinase together virtually eliminated the inhibition by iLac-CH_2Cl whereas either added alone had only a partial effect.

Since the effect of hexokinase was the most pronounced and reproducible, a study of its susceptibility to iLac-CH_2Cl was undertaken in a direct spectrophotometric assay of hexokinase. As shown in Figure 4, a pronounced difference in sensitivity to the inhibitor was observed when the enzyme from chick

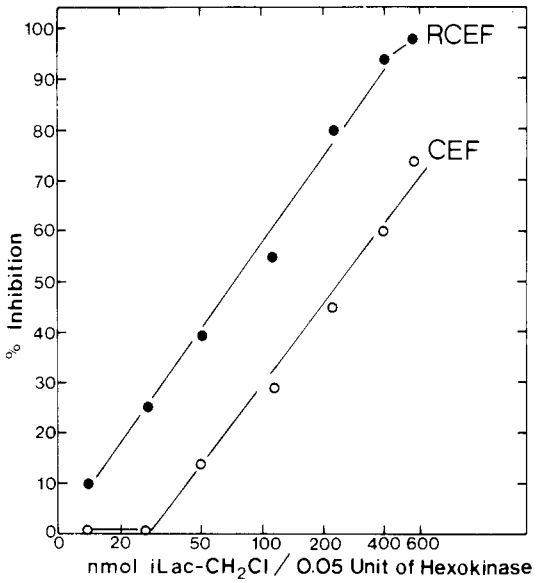

FIGURE 4: Inhibition of hexokinase activity in extracts from chick embryo fibroblasts (○) and Rous sarcoma virus transformed chick embryo fibroblasts (●) by iLac-CH_2Cl. Secondary cultures of CEF and RCEF cells were grown to confluency, harvested by removing the cells from the plates by trypsin treatment, and prepared from extracts as described under Experimental Procedures. Assays for hexokinase activity were performed spectrophotometrically as described under Experimental Procedures. The rates of glycolysis in control extracts were 0.25 μmol of lactate (30 min)$^{-1}$ (mg of protein)$^{-1}$ for CEF and 0.57 μmol of lactate (30 min)$^{-1}$ (mg of protein)$^{-1}$ for RCEF.

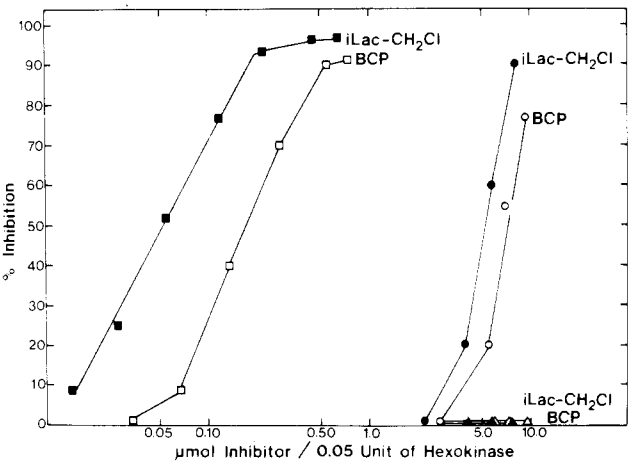

FIGURE 5: Inhibition of hexokinase from Ehrlich ascites tumor cells, mouse brain, and yeast by iLac-CH_2Cl and BCP. Extraction of enzymes and spectrophotometric assay of these enzymes are described under Experimental Procedures. Inactivation of both the Ehrlich ascites tumor cell enzyme and the mouse brain enzyme was independent of whether the enzymes were membrane bound or soluble. Data presented here are for the solubilized enzymes treated with iLac-CH_2Cl [EAT enzyme (■), mouse brain enzyme (●), and yeast enzyme ((▲)] and BCP [EAT enzyme (□), mouse brain enzyme (○), and yeast enzyme (△)]. Sample sizes were adjusted so that approximately 0.020 μmol of NADPH/min was formed for each hexokinase source. Actual activities of the enzymes were 0.024 μmol of NADPH min^{-1} (mg of protein)$^{-1}$ for the soluble tumor enzyme, 0.510 μmol of NADPH min^{-1} (mg of protein)$^{-1}$ for the mouse brain enzyme, and 187 μmol of NADPH min^{-1} (mg of protein)$^{-1}$ for the yeast enzyme.

embryo fibroblasts was compared with that from Rous sarcoma virus transformed cells. Considerable variability was, however, observed in these experiments. Sometimes the difference in sensitivity exceeded 10-fold, and at least a 3-fold difference in sensitivity was consistently observed. In Figure 5, experiments with Ehrlich ascites tumor, brain, and yeast

Table IV: Sensitivity of Brain and Ehrlich Ascites Tumor Hexokinase to 5,5′-Dithiobis(2-nitrobenzoate) and *N*-Ethylmaleimide[a]

inhibitors	% inhibition: tumor hexokinase, soluble	% inhibition: tumor hexokinase, particulate	% inhibition: brain hexokinase
5,5′-dithiobis(2-nitrobenzoate)			
2.5 μM	8	24	12
5 μM	44	86	60
10 μM	84	95	86
N-ethylmaleimide			
50 μM	18	22	12
250 μM	64	94	62
500 μM	90	97	84
1 mM	100	100	91

[a] 5,5′-Dithiobis(2-nitrobenzoate) and *N*-ethylmaleimide were added at the indicated concentrations and incubated at room temperature for 15 min. Assays were then performed as described under Experimental Procedures with sample sizes adjusted to form about 0.015 μmol of NADPH/min. Total activities of the samples were 0.020 μmol of NADPH min^{-1} (mg of protein)$^{-1}$ for the soluble tumor enzyme, 0.156 μmol of NADPH min^{-1} (mg of protein)$^{-1}$ for the particulate tumor enzyme, and 0.212 μmol of NADPH min^{-1} (mg of protein)$^{-1}$ for the particulate mouse brain enzyme.

Table V: Competition of 5,5′-Dithiobis(2-nitrobenzoate) for iLac-CH_2Cl and BCP Binding Sites with Hexokinase from Ehrlich Ascites Tumor Cells[a]

treatment	μmol of NADPH min^{-1} (mg of protein)$^{-1}$: EAT-soluble hexokinase, iLac-CH_2Cl	EAT-soluble hexokinase, BCP	EAT-particulate hexokinase, iLac-CH_2Cl	EAT-particulate hexokinase, BCP
none	0.020	0.020	0.180	0.180
DTNB (10 μM)	0.00	0.00	0.001	0.00
DTNB, dithiothreitol	0.015	0.016	0.16	0.115
DTNB, CH_2Cl, dithiothreitol	0.009	0.008	0.07	0.070
CH_2Cl, DTNB, dithiothreitol	0.001	0.001	0.003	0.00

[a] 5,5′-Dithiobis(2-nitrobenzoate) (10 μM), iLac-CH_2Cl (200 μM), or BCP (400 μM) was added to a sample containing 1 mg of protein/mL and incubated for 15 min at room temperature. After 15 min, the second addition was made as indicated and further incubated for 15 min at room temperature prior to assay. Thus the total preincubation time was 30 min. Where indicated, dithiothreitol (10 mM) was added 5 min before assay. Order of additions of these reagents is as indicated above, and assays were performed as described under Experimental Procedures.

hexokinase are included that show the different susceptibilities to both iLac-CH_2Cl and BCP. It can be seen that yeast hexokinase is completely resistant to the inhibitor while crude brain hexokinase preparations required 50–100-fold higher concentrations to show inhibition than are required for the Ehrlich ascites tumor enzyme. Since it was conceivable that a component present in the transformed cell extract conferred sensitivity to the inhibitor, a variety of mixing experiments were performed to demonstrate such sensitivity conferral. All were negative. Also, inactivation was not dependent upon whether the enzyme was membrane bound or soluble.

One of the most likely sites of interaction with the inhibitor was an SH group of hexokinase, and a comparison with other SH inhibitors was carried out. As shown in Table IV, both brain and tumor hexokinases were highly sensitive to 5,5′-dithiobis(2-nitrobenzoate) (DTNB) and *N*-ethylmaleimide. Although the particulate enzyme from Ehrlich ascites tumor

Table VI: Inhibition of Phosphorylation of Ehrlich Ascites Tumor Plasma Membranes and of Exogenous Substrates by 2-Isobutyl 3-Oxo-4-chloro-2-butyl Carbonate[a]

additions	pmol of ^{32}P (10 min)$^{-1}$ (mg of membrane protein)$^{-1}$: plasma membranes	+histone
none	199	285 (92)
iLac-CH_2Cl (100 nmol/mg)	135	158 (63)
iLac-CH_2Cl (200 nmol/mg)	102	101 (46)

[a] Protein kinase assays were performed in a final volume of 0.1 mL as described under Experimental Procedures except that casein and histones (100 μg of protein) were added to the membrane suspension where it is indicated. Inhibitors were incubated with the suspension for 15 min prior to the addition of [γ-^{32}P]ATP at 30 °C. The values in parentheses (average of two experiments) represent histone phosphorylation after removing the plasma membranes by centrifugation at 85000*g*.

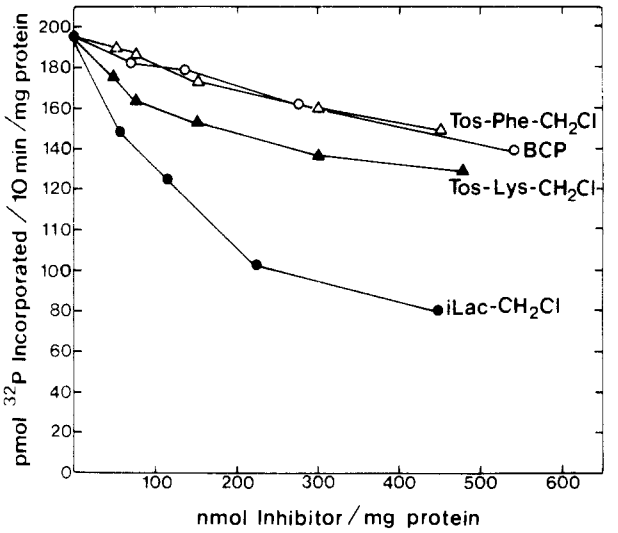

FIGURE 6: Inhibition of protein kinase activity in Ehrlich ascites tumor cell plasma membranes by iLac-CH_2Cl (●), BCP (○), Tos-Lys-CH_2Cl (▲), and Tos-Phe-CH_2Cl (△). Isolation of plasma membranes, treatment with inhibitors, and assays for protein kinase activity are described under Experimental Procedures.

cells was consistently somewhat more sensitive than either the soluble hexokinase or particulate brain hexokinase, the sensitivity was within the same order of magnitude. Since the inhibitions of enzymes by DTNB are often reversible, we explored the possibility that the chloromethyl ketones and DTNB interact at the same site. As shown in Table V, this seems to be the case. When iLac-CH_2Cl was added first, dithiothreitol had no effect. However, when DTNB was added before the chloromethyl ketone, followed by dithiothreitol, a marked protective effect by DTNB was observed both with iLac-CH_2Cl and with BCP in the case of soluble and particulate tumor hexokinase.

In view of the reported effects of Tos-Lys-CH_2Cl on protein kinase activity (Richert et al., 1979; Kupfer et al., 1979), the effects of iLac-CH_2Cl on the protein kinases of the ascites cell membranes were explored. As shown in Table VI, iLac-CH_2Cl inhibited, at concentrations that impaired glycolysis in intact cells, the phosphorylation of endogenous substrates, as well as of histones and casein added to the membrane. A comparison of the effect of different chloromethyl ketones is shown in Figure 6. Once again, iLac-CH_2Cl was the most effective in this series. At similar concentrations iLac-CH_2Cl had no effect on the activity of the catalytic subunit of cAMP-dependent protein kinase (kindly supplied by Dr. L. Pike), and

the changes observed in membrane phosphorylation in the presence of iLac-CH_2Cl were unaffected by the addition of dibutyryl-cAMP. However, tyrosine phosphorylation of the EGF receptor of A431 cell membranes in the presence of EGF was very sensitive (70% inhibition) to iLac-CH_2Cl whereas threonine protein kinase activity was not affected and serine protein kinase only mildly (15%).[3]

Discussion

Chloromethyl ketone derivatives of amino acids were first introduced as specific inhibitors of proteolytic enzymes [cf. Powers (1977)]. During our search for an inhibitor of lactic acid transport it was suggested to us by Dr. Arthur Pardee that a chloromethyl ketone derivative of lactic acid may serve in such a function. Although this did not turn out to be the case, a byproduct of the synthesis was a potent inhibitor (Johnson et al., 1980) of lactic acid transport. The chloromethyl ketone derivative was found to be a potent inhibitor of tumor cell glycolysis and cell growth.[1] The inhibition of glycolysis of cell-free extracts of Ehrlich ascites tumor cells was localized to both hexokinase and phosphofructokinase. However, while both inhibitions were readily prevented by cysteine, only phosphofructokinase activity was reversed by cysteine when the enzymes were incubated at room temperature. When the enzymes were exposed to iLac-CH_2Cl at 0 °C, then both activities were reversed by cysteine. These experiments indicate two modes of action by iLac-CH_2Cl: one leads to a reversible interaction between inhibitor and enzyme, and the second is an irreversible interaction as in the case of proteolytic enzymes.

We have focused on the inhibition of hexokinase because of a striking difference that was noted in the susceptibility of the tumor enzyme compared to that of the brain enzyme from the same animal or the yeast enzyme. Of particular interest, though less striking, is the difference in sensitivity between the hexokinase of virus-transformed cells and that of untransformed cells. A number of attempts to find an explanation for this difference have thus far been unsuccessful. Mixing experiments with crude brain and tumor enzyme preparation gave no indication for sensitivity conferral. In isoelectric focusing gel electrophoresis experiments stained for hexokinase activity (Scopes, 1968), no obvious differences were discovered (data not shown). One of the most attractive possibilities is posttranslational modification of the enzyme. In view of the recent advances in the identification of the *src* gene product as a protein kinase (Collett & Erikson, 1978; Levinson et al., 1978), the possibility of phosphorylation of hexokinase was considered, particularly since hexokinase activity was shown to be increased following transformation by Rous sarcoma virus (Fagan & Racker, 1978; Singh et al., 1974). Thus far, attempts to show such a mechanism have not been successful.

On the other hand, we have observed a pronounced inhibition of membrane-bound protein kinase activity of Ehrlich ascites tumor cells with both endogenous substrate and added protein substrates. Of particular interest is the effectiveness of the different chloromethyl ketone derivatives. iLac-CH_2Cl was the most effective inhibitor of both glycolysis and protein kinase activity. Further exploration of the structural and functional relationship of chloromethyl ketones, protein kinase activity, and glycolysis appears warranted.

[3] J. Navarro, M. Abdel-Ghany, and E. Racker, unpublished observation.

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